

A99  
**WO97040701**

**Publication Title:**

WO97040701

**Abstract:**

Abstract not available for WO97040701

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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> :  A23J 3/06	A1	(11) International Publication Number: WO 97/40701  (43) International Publication Date: 6 November 1997 (06.11.97)																																			
(21) International Application Number: PCT/US97/06605  (22) International Filing Date: 23 April 1997 (23.04.97)  (30) Priority Data: 08/641,463 1 May 1996 (01.05.96) US	(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.																																				
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(54) Title: CROSS-LINKED PROTEIN GELS AND METHODS OF MAKING THEM																																					
<table border="1"> <caption>Data points estimated from the graph</caption> <thead> <tr> <th>Time (min)</th> <th>Viscosity (cP) - Squares</th> <th>Viscosity (cP) - Triangles</th> <th>Viscosity (cP) - Circles</th> <th>Viscosity (cP) - Dots</th> </tr> </thead> <tbody> <tr><td>0</td><td>10</td><td>10</td><td>5</td><td>10</td></tr> <tr><td>60</td><td>50</td><td>10</td><td>5</td><td>10</td></tr> <tr><td>90</td><td>150</td><td>10</td><td>5</td><td>10</td></tr> <tr><td>100</td><td>1500</td><td>10</td><td>5</td><td>10</td></tr> <tr><td>230</td><td>-</td><td>10</td><td>10</td><td>-</td></tr> <tr><td>280</td><td>-</td><td>800</td><td>-</td><td>-</td></tr> </tbody> </table>			Time (min)	Viscosity (cP) - Squares	Viscosity (cP) - Triangles	Viscosity (cP) - Circles	Viscosity (cP) - Dots	0	10	10	5	10	60	50	10	5	10	90	150	10	5	10	100	1500	10	5	10	230	-	10	10	-	280	-	800	-	-
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(57) Abstract																																					
<p>Enzymatically cross-linked protein gels and methods for preparing them are disclosed. The methods comprise adding a transglutaminase, such as factor XIII, to a composition of a temperature-sensitive gel-forming protein, such as gelatin or collagen, and incubating the composition and transglutaminase under gel-forming conditions. The resulting gels have superior strength and thermal stability, and can be used within a variety of medical and industrial applications.</p>																																					

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Description

## 5      CROSS-LINKED PROTEIN GELS AND METHODS OF MAKING THEM

Background of the Invention

Protein gels are used in a variety of applications in industry and medicine. For example, 10 gelatin films are used for photographic emulsions. In the food industry, protein gels are components of processed fish products ("surimi"), candies, desserts, fat substitutes, soy products and milk products. See, for example, WIPO publication No. WO 93/22930; Japanese 15 Patent Application Publication No. 5260893; and U.S. Patent No. 5,420,025. In medicine, gels are used as delivery vehicles, such as gelatin capsules (e.g., published French Patent Application No. 3 659 352 A1) and vehicles for topical application of polypeptide or other 20 drugs (e.g., U.S. Patent No. 5,427,778). Gels are also used in cosmetic preparations.

Conventional protein gels often lack thermal stability, high strength, resistance to proteolysis after enzymatic cross-linking (McDonagh, J., in Hemostasis and 25 Thrombosis, 2nd Ed., ed. C. Colman et al., J.B. Lippincot Co., Philadelphia, 1987), and elasticity, thus limiting their use. For example, conventional gelatin gels melt at about 28-32°C, depending on protein concentration. This melting is reversible, with the solution converting 30 back to the gel state upon lowering of the temperature. Concentration dependence of gelation and other properties of gelatin has been reported by Michon et al. (Rheol. Acta 32:94-103, 1993).

Stabilization of gels can be achieved by cross-linking, either by chemical or enzymatic means. See, for 35 example, Nastruzzi et al., J. Microencapsulation 11(3):249-260, 1994; Digenis et al., J. Pharm. Sci.

83(7):915-921, 1994; Shinde et al., Bio-Medical Materials and Engineering 2:123-126, 1992; van Washem et al., J. Biomed. Mater. Res. 28:353-363, 1994; Koide et al., J. Biomed. Mater. Res. 27:79-87, 1993; and Penhoat et al., 5 Biomaterials 14(7):503-506, 1993. Enzymatic cross-linking of proteins in complex mixtures (food products) using transglutaminases has been reported (e.g., U.S. Patent No. 4,917,904). Such methods, which involve incubation of enzyme-substrate mixtures at temperatures up to 90°C, are reported to improve food texture. 10 Chemical cross-linking, such as with glutaraldehyde or carbodiimide, improves gel strength but produces an amorphous (non-uniform) gel. See, for example, Gerhart et al., J. Biomed. Mater. Res. 22(11):1071-1082, 1988; 15 and Kaalem et al., Nature 325(6102):328-329, 1987. Chemical cross-linking agents are often toxic, immunogenic, and/or inflammatory, thereby limiting their use in the production of foodstuffs, medicaments, and cosmetics.

20 Gels are a thermodynamic state and, as such, their properties at equilibrium should be independent of the pathway taken for the liquid/gel phase transition. In practice, however, gel properties are dependent on the kinetics of gelation, and the kinetics are in turn a 25 function of the pathway. Furthermore, it is possible to force molecules into a qualitatively different physical state though the use of crosslinking agents, such as chemical cross-linking agents and transglutaminases. For example, gelatin or other protein solutions can be 30 crosslinked before they can naturally gel and/or under conditions where they would not otherwise gel at all (e.g., high temperature). Gelatin can thus be forced to form such networks (sometimes referred to as gels) at temperatures above its gel point. Although such cross-linking results in a reduction in solubility and an - 35 increase in working temperature, the resulting materials

may have even less strength and elasticity than conventional gels, and native protein structure (and biological properties) may be lost.

There remains a need in the art for high-strength, uniform protein gels. There also remains a need for improved protein gels that are free of toxic agents. There is an additional need for protein gels with improved thermal stability. There is a further need for methods for incorporating additives, such as bioactive molecules, into gels. The present invention provides such improved gels and methods of making improved gels, as well as other, related advantages.

#### Summary of the Invention

Within one aspect, the invention provides a method of preparing a cross-linked protein gel comprising the steps of (a) adding a transglutaminase to a composition of a temperature-sensitive gel-forming protein; and (b) incubating the composition and transglutaminase at a temperature at which a like composition lacking transglutaminase will gel, whereby the composition is converted to a cross-linked gel. Within one embodiment, the composition is in the form of an aqueous solution. Within an alternative embodiment, the composition is in the form of a gel. Within additional embodiments, the gel-forming protein is gelatin or collagen. Within other embodiments, the transglutaminase is selected from the group consisting of factor XIII, tissue transglutaminase, keratinocyte transglutaminase, epidermal transglutaminase, and prostate transglutaminase. The composition may further comprise one or more additional proteins selected from the group consisting of fibronectin, von Willebrand factor, vinculin, and laminin. The composition may also further comprise a cytokine or a hormone.

Within a second aspect of the invention there is provided a method of preparing a cross-linked protein gel comprising the steps of (a) forming an aqueous solution of a temperature-sensitive gel-forming protein, 5 (b) adding transglutaminase to the solution in an amount sufficient to catalyze cross-linking of the temperature-sensitive gel-forming protein, and (c) incubating the transglutaminase-containing solution under conditions of time and temperature in which a like solution lacking 10 transglutaminase will gel. Within a preferred embodiment, the aqueous solution is subjected to at least one cycle of gelation and melting before the addition of the transglutaminase. Within another embodiment, the protein is gelatin and the transglutaminase-containing 15 solution is incubated at a temperature of from 4°C to 33°C. Within other embodiments, the gel-forming protein is gelain or collagen. Within an additional embodiment, the protein is gelatin, the gelatin concentration is from 1% to 10% by weight, and the transglutaminase-containing solution is incubated at a temperature of from 20°C to 20 32°C. Within a further embodiment, the protein is gelatin, the transglutaminase is factor XIII, and the gelatin and factor XIII are present in a mass ratio of from 528:1 to 66:1. The transglutaminase-containing 25 solution may further comprise one or more additional proteins selected from the group consisting of fibronectin, von Willebrand factor, vinculin, and laminin, preferably in an amount not exceeding 10% by weight of said gel. The transglutaminase-containing 30 solution may also further comprise a cytokine or a hormone.

Within a third aspect of the invention there is provided a method of preparing a cross-linked protein gel comprising adding a transglutaminase to a protein gel, whereby the transglutaminase diffuses into the gel and 35 catalyzes cross-linking of the protein.

Within a fourth aspect of the invention there is provided a cross-linked protein gel prepared according to one of the methods disclosed above. Within one embodiment, the gel is in the form of a pharmaceutical composition, such as a composition in which the gel provides a vehicle for an amine-containing drug, wherein the drug is cross-linked to the gel. Within a related embodiment, the gel is in the form of a microcapsule. Within another embodiment the gel is coated on a polymeric or paper support and may further comprise silver halide grains or a dye-forming coupler compound.

These and other aspects of the invention will become evident upon reference to the following detailed description and the attached drawings.

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#### Brief Description of the Drawings

Fig. 1 illustrates changes in viscosity of gelatin gels prepared under various conditions as disclosed in Example 1.

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Fig. 2 illustrates the relationship between gelatin concentration and tensile strength.

#### Detailed Description of the Invention

The term "cross-linking" is used herein to denote the formation of covalent bonds between polymer chains to make a single network with increased strength and resistance to solvents. Transglutaminases catalyze the formation of  $\gamma$ -glutamyl  $\epsilon$ -lysine isopeptide bonds.

As used herein, the term "factor XIII" includes the complete factor XIII zymogen tetramer ( $A_2B_2$ ) and factor XIIIa ( $A'2$  or  $A'A$ ), as well as activation intermediates and subunits thereof, including  $A'_2B_2$  and  $AA'B_2$  intermediates, the  $A$  subunit, the  $A'$  subunit and  $A_2$  dimers. The  $A_2$  dimer, which is readily produced by recombinant methods or can be isolated from platelets and placenta, is a preferred form.

The term "gel" is used herein to denote a swollen, hydrated polymer network which is essentially continuous throughout its volume. A protein gel is composed of an essentially continuous network of linked 5 protein molecules and a liquid (typically aqueous) solvent, which fills the space within the protein matrix. The protein matrix exerts a strong viscous drag on the solvent molecules, preventing them from flowing freely. The component molecules making up the gel 10 network may be linked by ionic, hydrophobic, metallic or covalent bonds. The covalent bond is the most thermally stable of these bonds.

The term "gel point" is used for the transition point between the fluid (sol) state and the gel state. 15 The gel point can be observed as a sudden loss of fluidity as the material rapidly changes from a viscous liquid to an elastic material of infinite viscosity. This sudden, discontinuous transition in the viscosity of the fluid is marked by changes in physical 20 characteristics; bubbles no longer rise in the material, and breaking strength and elastic modulus increase. At the gel point the molecular weight (weight average) of the protein matrix becomes "infinite", i.e., an essentially continuous matrix is formed throughout the 25 nascent gel. Polymerization can continue beyond this point, incorporating more soluble protein into the matrix.

A "temperature-sensitive gel-forming protein" 30 is a protein that gels in response to a change in temperature. These proteins have a definable gel point, the temperature at which gelation occurs. The gel point of a protein varies somewhat with protein concentration and other factors. Examples of temperature-sensitive 35 gel-forming proteins include gelatin and collagen. Gelation of these proteins is discussed in more detail below. This definition specifically excludes proteins

that form gel-like matrices in response to other physical changes, such as the formation of fibrin polymers upon proteolysis of fibrinogen. The term "temperature-sensitive protein gel" is used to denote gels formed from 5 temperature-sensitive gel-forming proteins.

The present invention is based in part upon the discovery that enzymatically cross-linking protein gels during or subsequent to gelation results in gels having superior properties, including uniformity, strength, and 10 thermal stability. According to the present invention improved gels are formed by enzymatically cross-linking protein compositions under conditions in which a like composition lacking cross-linking enzyme will gel. Thus, a transglutaminase is added to a composition of a 15 temperature-sensitive gel-forming protein, such as gelatin or collagen, and the composition and transglutaminase are incubated at a temperature at which a like composition lacking transglutaminase will gel, whereby the composition is converted to a cross-linked 20 gel. Within one embodiment of the invention, a solution of protein and transglutaminase is incubated at a temperature conducive to gel formation and for a time sufficient for gel formation. The inventors have found that enzymatic cross-linking under gel-forming conditions 25 results in improved strength and thermal stability as compared to non-cross-linked gels or gels subjected to enzymatic cross-linking under sol-state conditions. For example, gelatin gels produced according to the methods of the present invention using factor XIII as the cross-linking enzyme are, on average, more than 2.5 times as strong as comparable gels prepared without factor XIII. Cross-linking stabilizes the higher order structure of 30 protein gels, making them stable at temperatures above the melting point of otherwise similar, non-cross-linked gels. Such stabilization is also expected to make the 35 cross-linked gels resistant to proteolysis in view of the

known relationship between helical structure and protease resistance (see Raghunath et al., J. Mol. Biol. 236:940-949, 1994).

Experimental evidence also indicates that gels prepared according to the present invention are cross-linked primarily at native helix formation sites (i.e., adhesion sites that are zones of partially renatured helix). See, Ross-Murphy, Polymer 33: 2622 - 2627, 1992; and Stainsby, G., in Food Polymers, Gels and Colloids, ed. E. Dickinson, Royal Society of Chemistry, London, 1991. Cross-links produced by transglutaminases are contrasted to chemically-produced cross-links in that they selectively stabilize the collagen-like helix structure of adhesion zones. Transglutaminase cross-linking thereby provides for a more normal and uniform gel structure, whereas chemically cross-linked gels exhibit a more random pattern of cross-linking. While not wishing to be bound by theory, it is believed that the observed pattern of enzymatic cross-linking results from the formation of at least most of the cross-links after gelation has occurred.

Cross-linking of protein gels increases their thermal stability. However, melting of cross-linked gels is irreversible insofar as the improved properties are lost. The present inventors have discovered, however, that gelatin gels cross-linked with a representative transglutaminase, factor XIII, according to the methods of the present invention are stable to above 100°C. Thermal stability (melting temperature) of such gels is a function of degree of cross-linking, which can be controlled by adjusting the cross-linking time by, for example, thermal or chemical denaturation of the transglutaminase or by adding selective inhibitors prior to completion of cross-linking, or by adjusting the transglutaminase concentration.

Transglutaminases useful within the present invention include those produced by microorganisms and higher organisms. Mammalian transglutaminases, such as factor XIII (U.S. Patent No. 5,204,447; European Patent No. 0 268 772), keratinocyte transglutaminase (Phillips et al., Proc. Natl. Acad. Sci. USA 87:9333-9337, 1990; Phillips et al., Biochemistry 32:11057-11063, 1993), tissue transglutaminase (WIPO Publication WO 92/12238), prostate transglutaminase (WIPO Publication WO 93/13207), and epidermal transglutaminase (WIPO Publication WO 91/06553; Folk, Ann. Rev. Biochem. 49:517-531, 1980), are preferred. Microbial transglutaminases can also be used, particularly in the preparation of food products. Preferred microbial transglutaminases include those from fungi and bacteria, a variety of which are known in the art. Transglutaminases can be obtained from, for example, *Streptoverticillium* species, such as *Streptoverticillium mobaraense* (U.S. Patent No. 5,156,956); *Streptomyces lydicus*, *Cercospora*, and *Bacillus* (WIPO Publication WO 96/06931); and Oomycetes, including *Phytophthora cactorum* (WIPO Publication WO 96/22366). Additional microbial transglutaminases are disclosed in European Patent Office Publication 0 379 606 A1 and U.S. Patent No. 5,420,025. For medical uses, it is preferred to use a transglutaminase from the species to be treated so as to avoid complications due to immunogenicity. Medical products intended for use in humans are preferably made using a human transglutaminase, while veterinary products are prepared using an appropriate non-human transglutaminase.

Transglutaminases for use within the present invention are prepared according to known means, including microbial fermentation (U.S. Patent No. 5,156,956), extraction from plasma or tissue (Cooke and Holbrook, Biochem. J. 141: 79-84, 1974; Curtis and Lorand, Methods Enzymol. 45: 177-191, 1976; U.S. Patents

Nos. 3,904,751; 3,931,399; 4,597,899; 4,285,933; and 5,130,879), or recombinant production. Recombinant production (i.e., production using genetically engineered host cells) is preferred because it allows production of highly purified proteins uncontaminated by infectious agents and can be scaled up for commercial production. Methods for preparing recombinant transglutaminases are disclosed by, for example, Bishop et al., U.S. Patent No. 5,204,447; Davie et al., EP 268,772; Grundmann et al., AU-A-69896/87; and WIPO Publications WO 91/06553, WO 92/12238, and WO 93/13207, which are incorporated herein by reference in their entirety.

It is preferred to produce factor XIII A<sub>2</sub> dimer cytoplasmically in yeast (*Saccharomyces cerevisiae*) using the method disclosed by Bishop et al. (Biochemistry 29: 1861 - 1869, 1990), in copending United States Patent Application Serial No. 08/333,236, and in WIPO Publication WO 93/03147, which are incorporated herein by reference in their entirety. The cells are harvested and lysed, and a cleared lysate is prepared. The lysate is fractionated by anion exchange chromatography at neutral to slightly alkaline pH using a column of derivatized agarose, such as DEAE Fast-Flow Sepharose™ (Pharmacia) or the like. Factor XIII is then crystallized from the column eluate by concentrating the eluate and adjusting the pH to 5.2-5.5, such as by diafiltration against ammonium succinate buffer. The precipitate is then dissolved and further purified using conventional chromatographic techniques, such as gel filtration and hydrophobic interaction chromatography.

Temperature-sensitive gel-forming proteins that can be used within the present invention include gelatin, collagen, casein, whey proteins (e.g., alpha-lactalbumin), and synthetic polymers containing transglutaminase substrate sites (e.g., polymers disclosed in U.S. Patent No. 5,428,014). Such synthetic

polymers will generally comprise repeating polypeptide units, including transglutaminase substrate sites, such as the Leu-Ser-Gln-Ser-Lys sequence of  $\beta$ -casein (SEQ ID NO:1). Gels can also be formed between mixtures of temperature-sensitive gel-forming proteins. For example, the gelation of a heat-induced complex between  $\kappa$ -casein and  $\alpha$ -lactalbumin is disclosed by Doi et al. (J. Nutr. Sci. Vitaminol. (Tokyo) 31:77-87, 1985).

An exemplary protein for use in preparing a gel according to the present invention is gelatin. Gelatin is produced from collagen by acid (Type A) or alkaline (Type B) hydrolysis and thermal denaturation of the collagen fibers (Ross-Murphy, *ibid.*). With heating, the triple helix of the hydrolyzed collagen unfolds, and the protein becomes soluble. Cooling of the protein, now referred to as gelatin, causes a partial re-folding of the helix and results in a network of helical junctions. At a critical point determined by such factors as protein concentration, temperature, and ionic strength of the solvent, the network is extensive enough to form a gel. These gels are thermoreversible gels, and their properties have been extensively studied. See Michon et al. (*ibid.*) and references cited therein.

Those skilled in the art will recognize that the methods of the present invention can also be applied to other temperature-sensitive gel-forming proteins. A preferred such other temperature-sensitive gel-forming protein is collagen. Collagen occurs in fibrillar and non-fibrillar forms. In the latter, the Gly-X-Y collagen repeat sequence is interrupted by other sequences. The length and extent of these other sequences may affect the thermal stability of the protein. Single triple helical collagen molecules can be extracted by any of several methods. See, for example, Miller and Rhodes, "Preparation and Characterization of the Different Types of Collagen" in Cunningham and Drederiksen (eds.),

Methods Enzymol. 82, part A:33-62, 1982; Cheung et al., J. Biol. Chem. 258:7774-7778, 1983; Mayne and Zettergren, Biochemistry 19:4065-4072, 1980; Carmichael et al., Biochim. Biophys. Acta 491(1):177-192, 1977; Bishop et al., Biochem. Biophys. Res. Comm. 185:392-397, 1992; and U.S. Patent No. 4,703,108. Gelation of collagen occurs when the individual molecules aggregate and form fibrils (Djabourov et al., Biorheology 30:191-205, 1993). Collagen remains soluble in acid at low temperature and ionic strength. The rate of gelation of collagen increases with increasing temperature to an optimum temperature that is dependent in part on the source species, type of collagen, protein concentration, pH, and ionic strength. Collagens from mammalian species typically gel within a range of about 25°C to 37°C. For example, an aqueous solution of bovine fibrillar collagen (commercially available from Collagen Corporation, Fremont, CA) is adjusted to isotonicity and near-neutral pH (7.4 ± 0.2). The resulting solution can be stored at 4-6°C for several hours. Gelation (fibrillogenesis) is initiated by warming the solution to 37°C for at least about 60 minutes. The temperature range within which collagen gels are stable varies with the type of collagen and the species. The amounts of proline and hydroxyproline residues and their positions within the collagen molecules are primarily responsible for this variation in temperature stability. Variation is believed to have arisen in response to environmental factors such as habitat temperature and pressure (Har-El and Tanzer, FASEB J. 7:1115-1123, 1992). As described in more detail below, transglutaminase is added to the collagen solution prior to or during gelation, or may be applied to the surface after gelation is substantially complete. Thus, the methods and compositions comprising gelatin described below are representative of certain embodiments of the invention, and can be readily adapted

for use with other temperature-sensitive gel-forming proteins.

Within certain embodiments of the invention, gelatin is prepared as an aqueous solution. It is preferred to dissolve gelatin in an aqueous solvent buffered at pH 5.5-9.0 and of low to moderate ionic strength (equivalent to about 1 to 1000 mM NaCl, preferably 100 to 150 mM NaCl). While salt is not required in the buffer, certain proteins are more soluble in the presence of salt, and near-physiological concentrations of salt are beneficial in compositions that are to be used within living organisms. Preferably the pH of the solution is about 6.0-8.0, more preferably about 7.4. A preferred aqueous solvent in this regard is phosphate buffered saline (PBS; 10 mM sodium phosphate pH 7.4, 120 mM NaCl, 2.7 mM KCl). Other suitable buffers include borate, phosphate, HEPES (N-[2-Hydroxyethyl]piperazine-N'-(2-ethanesulfonic acid)) and the like. The concentration of gelatin will be determined according to its intended use, but will generally be 20% or less by weight, more commonly 1% - 10%. It is convenient to prepare a concentrated solution (e.g., a 10% stock solution) that can be diluted to the desired working concentration immediately prior to gelation and cross-linking. The gelatin is allowed to swell, typically for about two hours, then melted by raising the temperature to above the gel point of the solution, preferably to at least 32°C up to 100°C or more, more preferably to 32°C to 60°C. Use of temperatures above 60°C significantly increases the time required for gelling upon cooling of the solution. The solution can then be gelled, such as by allowing it to stand overnight at room temperature, and stored refrigerated until needed. If stored in the solid state, the gel is melted immediately before use by heating (typically to 37°C) and diluted to the desired working

concentration (commonly about 2% to 6.6% weight/volume). While not necessary for the realization of certain benefits of the invention, the solidification and remelting of the solution has been found to improve the rate of gelation and may therefore reduce the amount of transglutaminase needed to obtain the desired degree of cross-linking. Large amounts of transglutaminase can cause deamidation, rather than cross-linking, of glutamine residues in the protein.

Transglutaminase is then added to the solution of gelatin in the sol state. Depending upon the transglutaminase used, the solution may also contain sufficient calcium (1-18 mM, preferably about 3 mM, as CaCl<sub>2</sub>) for transglutaminase activity. Mammalian transglutaminases, including factor XIII, tissue transglutaminase, keratinocyte transglutaminase, epidermal transglutaminase, and prostate transglutaminase, require Ca<sup>++</sup> for activity, while others, including certain bacterial transglutaminases (see, e.g., European Patent Office Publication 0 379 606 A1; U.S. Patent No. 5,420,025), do not require Ca<sup>++</sup>. When using factor XIII zymogen as the transglutaminase, thrombin (0.1 - 10 U/ml, preferably about 1 U/ml) is included for activation of the factor XIII. Transglutaminases may also be pre-activated (e.g., factor XIIIa) or a transglutaminase not requiring activation can be used (e.g., tissue transglutaminase or keratinocyte transglutaminase). The ratio of gelatin:transglutaminase will generally be within the range of 1320:1 to 20:1 by weight, preferably 528:1 to 66:1, more preferably 264:1 to 66:1. The concentration of transglutaminase within the solution will typically be from 0.125 mg/ml to 2.0 mg/ml, preferably at least 0.5 mg/ml, although the concentration can be adjusted to provide gels with low melting points or very high or low tensile strength. The solution is then incubated under conditions of time and

temperature in which a like solution lacking transglutaminase will gel. Gelatin-transglutaminase solutions are incubated at from about 4°C up to the gel point, preferably from 4°C to 33°C, more preferably at a 5 temperature of at least 20°C up to about 32°C. Those skilled in the art will recognize that time to gel formation is a factor of temperature and protein concentration, and the actual time and temperature of incubation will be determined with consideration of all 10 relevant parameters. For example, the critical gelation point for 4% (w/v) Type A gelatin is 29.1°C (Michon et al., ibid.), and a 5% gelatin solution will gel in 260 minutes at 26°C.

Within an alternative embodiment of the 15 invention, transglutaminase can be added to a composition of a temperature-sensitive gel-forming protein during or subsequent to gelation, in the latter case allowing the transglutaminase to diffuse into the gel. For example, an aqueous solution of transglutaminase is applied to the 20 surface of a newly-formed collagen or gelatin gel and allowed to diffuse into the gel. The gel and transglutaminase are incubated under conditions conducive to gel formation for the protein used within the gel. For example, a factor XIII solution is applied to the 25 surface of gelatin gel and incubation is carried out at below the melting point of the gelatin, preferably about 20°C to 32°C. Diffusion time, and amount and concentration of transglutaminase can be adjusted to provide the desired depth of penetration and degree of 30 cross-linking. This alternative method is particularly suited to cross-linking gels that are sufficiently thin to allow the transglutaminase to diffuse throughout the gel; to produce thin, cross-linked membranes; or to harden surfaces of thicker gel products, including 35 foodstuffs wherein a soft center surrounded by a harder coating is desired. Within one method for preparing thin

membranes, after the desired degree of penetration and cross-linking has occurred, the gel is heated to above the melting point of the non-cross-linked gel, and melted protein is separated from the remaining membrane.

5        Additional proteins can also be incorporated into gels of the present invention. It is known in the art that transglutaminases will catalyze the formation of bonds between a glutamine-containing peptide and a peptide containing an  $\epsilon$ -lysine amine. It is therefore  
10      possible to cross-link most proteins containing susceptible lysine or glutamine residues into a protein gel if the gel-forming protein contains a suitable complementary residue. Suitable additional proteins in this regard include fibronectin, vinculin, von Willebrand  
15      factor, laminin, fibrin, and various hormones and cytokines, including, for example, the AA, BB, and AB isoforms of platelet derived growth factor (PDGF), transforming growth factors  $\alpha$  and  $\beta$  (TGF- $\alpha$  and TGF- $\beta$ ), and acidic and basic fibroblast growth factors (aFGF and bFGF). Methods for preparing these proteins are known in  
20      the art. See, for example, Lynch et al., Cell 41:49-56, 1985; Chopek et al., Biochemistry 25:3146-3155, 1986;  
Obara et al., Cell 53:649-657, 1988; Asijee et al., Biochim. Biophys. Acta 954:303-308, 1988; Dufour et al.,  
25      EMBO J. 7:2661-2671, 1988; Brubacher et al., Exp. Cell Res. 197:290-299, 1991; Brandenberg and Chiquet, J. Cell Science 108:3099-3108, 1995; U.S. Patents Nos. 4,703,108; 4,801,542; 4,766,073; 4,902,782; 5,104,977; 5,155,214; and WIPO Publication WO 95/23868). Additional proteins  
30      are included in the gel in an amount not exceeding 50% by weight of the total gel protein. It is preferred that the amount of additional protein not exceed 10% by weight. It is particularly preferred that additional proteins, when included, be present at 1%-5% by weight, although for certain proteins and applications the content will be substantially less.  
35

The gels of the present invention can also comprise amine-containing, non-proteinaceous compounds. Examples of such compounds are the polyamines spermidine, cadaverine, and putrescine, which can provide attachment sites for cells; and bis-amine polyethylene glycol, which can be included to modify gel structure.

The gels of the present invention can be used in industrial and medical applications in place of conventional cross-linked and non-cross-linked gels. Such applications include photographic films, gelatin-containing foodstuffs, gelatin capsules (particularly microcapsules), drug delivery devices, and prosthetic devices. The enzymatically cross-linked gels of the present invention provide the advantages of uniformity and specific cross-linking in combination with high thermal stability, which permit use of the gels within new and expanded applications.

The methods of the present invention can be used in the preparation of photographic emulsions. Photographic emulsions are typically gelatin-based emulsions containing one or more of silver halide grains, dye-forming coupler compounds, ultraviolet absorbing materials, developing agents, etc. These compounds are added to the gelatin to form a mixture which is applied to a polymeric film base or paper surface in the sol state and allowed to gel and cross link. Surfaces can be coated with single or multiple layers of emulsion as desired. Methods for preparing photographic emulsions and applying them to surfaces are known in the art. See, for example, U.S. Patents Nos. 3,607,345; 4,214,047; and 4,269,927, which are incorporated herein by reference in their entirety.

The methods of the present invention can also be used in the preparation of protein-containing food products, for example to improve the functional properties of proteinaceous foods. Within the present

invention, transglutaminase is added to such foodstuffs as minced meat, fish paste, milk, gelatin, whey protein, soy protein, wheat protein, maize protein, egg albumin, rape seed protein, potato protein, and the like. The 5 resulting mixture is incubated for a period of time sufficient for gelation to occur and for the transglutaminase to react with the substrate. Foodstuffs prepared in this manner exhibit increased water binding capacity, improved texture, improved thermal stability, 10 and other advantages. The resulting alteration in the functional properties can be utilized to lower the fat content in the food product, because the crosslinked protein simulates properties associated with a higher fat content. The methods of the present invention are 15 particularly useful in the preparation of food products rich in gelatin or collagen, such as gelatin desserts and collagen sausage casings.

The methods of the present invention can be used to prepare drug delivery vehicles such as 20 microcapsules. For example, gelatin microcapsules can be prepared by complete coacervation (Thies, Crit. Rev. Biomedical Engineering 8: 335-383, 1982; incorporated herein by reference). Two oppositely charged 25 polyelectrolytes (gelatins type A and B) are combined in an aqueous solution to form a coacervate which also includes the drug to be encapsulated. During gelation, the mixture is vigorously agitated to form small beads, which are cross-linked by transglutaminase as disclosed herein, making them relatively insoluble, yet 30 biodegradable. In the alternative, microcapsules can be formed from organic/aqueous emulsions according to known methods and subsequently cross-linked. Microcapsules containing both peptidic and non-peptidic drugs can be prepared by this method. In a related embodiment, drugs - 35 containing amines, including proteins and peptides containing lysine residues, can be covalently bonded to

microcapsules during the hardening process. Amine-containing substances can be added to the gel prior to or during cross-linking, whereby the amines become covalently bonded to glutamine residues in the gel-forming protein by the action of the transglutaminase. Microcapsules of this type can be used to provide localized or timed-release delivery of drugs. Drugs that can be delivered in this way include hirudin, glucagan, insulin, protamine, amphotomycin, and bacitracin.

The gels of the present invention can also be used in prosthetic devices, for example to promote the adhesion of vascular endothelial cells seeded on prosthetic vascular grafts. Methods for seeding and sodding vascular grafts with endothelial cells are disclosed by, for example, Budd et al., Br. J. Surg. 76:1259-1261, 1989; Kaehler et al., J. Vasc. Surg. 9:535-541, 1989; Mosquera and Goldman, Br. J. Surg. 78:656-660, 1991; WIPO Publication WO 91/16009; and Rubens et al., U.S. Patent No. 5,324,647. Vascular endothelial cells attach and spread when their cell membrane integrins contact and bind to matrix components such as fibronectin, laminin, and von Willebrand factor. In the case of fibronectin the N-terminus contains a transglutaminase substrate site that allows it to be cross-linked to a gelatin or collagen matrix. See Bockenstedt et al., J. Clin. Invest. 78:551-556, 1986. The C-terminal portion of fibronectin contains the ligand for binding to the  $\alpha 5\beta 1$  integrin. Properly oriented fibronectin will act as a tether for endothelial cells and could be used to reduce sluffing of seeded endothelial cells. Other proteins that provide contact points for cell attachment and/or migration and cytokines can also be included. Polyamines, such as spermidine, cadaverine, and putrescine can also be included to provide cell-attachment points.

Protein gels can be shaped by casting (forming the gel within a mold while in a plastic state) or by extruding during or after cross-linking to produce a fiber that can be woven. Additional compounds can be 5 incorporated into or onto such gels. For example, amine-containing compounds can be bound to a surface of a prosthetic material to provide a transglutaminase substrate site. Methods of binding include treatment with a strong oxidant, such as potassium permanganate, 10 nitric acid, dichromates, or peroxides; cross-linking; and adsorption.

Cross-linked gels of the present invention can be used in surgical applications where non-cross-linked gels are currently used, such as in neural reconnective 15 surgery to prevent adhesions. Polymeric gels of this type are known in the art (e.g., Adcon®, produced by Gliatech Inc., Cleveland, OH). Cross-linking stabilizes the gel, increasing its residence time.

Enzymatically cross-linked gels are 20 particularly well suited for *in vivo* uses in view of the protease resistance of stabilized helices and the improved thermal stability of the gels. Gels having a melting point of 37°C or above can be prepared, with the actual melting point depending on the degree of cross-linking. It is thus possible to prepare gels that are 25 stable at, for example, 45°C, 65°C, or 100°C.

The invention is further illustrated by the following non-limiting examples.

30

### Examples

#### Example 1

All viscosity measurements were taken at 37°C in a jacketed Brookfield DV III cone and plate viscometer with a cp40 cone (Brookfield Engineering Labs, -35 Stoughton, MA).

Human factor XIII A<sub>2</sub> dimer was produced in *S. cerevisiae* and stored as a lyophilized powder containing 13.2 mg factor XIII, 0.66  $\mu$ Moles EDTA, 6.60  $\mu$ Moles glycine, and 0.13 grams sucrose. Prior to use, the 5 lyophilized material was dissolved in 1 ml of distilled water.

Calf skin Type A gelatin, high Bloom strength (Ferrosan International A/S, Soeborg, Denmark) was dissolved in Tris-buffered saline (TBS: 25 mM Tris pH 10 7.4, 120 mM NaCl, 0.2% NaN<sub>3</sub>) containing 3 mM CaCl<sub>2</sub>. A 10% w/v stock solution was allowed to swell at room temperature for two hours. The gelatin was dissolved by heating at 37°C, and the solution was allowed to gel overnight at 4°C.

15 The 10% w/v gelatin stock was melted at 37°C and diluted to 4% w/v with TBS + CaCl<sub>2</sub>, and 1-ml aliquots were dispensed into 13 100-mm plugged test tubes. The experiment was initiated by adding 1 mg recombinant human factor XIII and 10 units bovine thrombin (Enzyme Research 20 Laboratories, West Bend, IN) to each tube. The tubes were incubated at 27°C to allow gelling of the solution, and viscosity measurements were taken at intervals until the viscosity exceeded the range of the instrument (290 minutes). For each time point during gelation an 25 aliquot was incubated for five minutes at 37°C, and a second aliquot was incubated at 100°C. The viscosities of both aliquots were then measured at 37°C after an equilibration period of five minutes. Control samples containing no factor XIII were melted and measured at 30 37°C only.

As shown in Fig. 1, the viscosity of the control containing no factor XIII (>) increased only slightly over the entire coarse of the experiment. Samples containing factor XIII incubated at 100°C (•, Δ) exhibited a slow increase in viscosity for about 250 minutes, then viscosity increased exponentially until 35

going off the scale after the 290 minute time point. Factor XIII- containing samples incubated at 37°C (■) showed an almost immediate increase in viscosity, which after only 60 minutes reached a point equivalent to the 5 viscosity of the 270 minute time point in the samples incubated at 100°C.

The differences in viscosity between the 37°C and 100°C samples are interpreted as indicating a loss of structure at the higher temperature for reaction times 10 shorter than 250 minutes. The small but continuous increase in the viscosity of the 100°C samples suggests that some cross-linking is occurring at times below 250 minutes. The increase in viscosity seen below 250 minutes in the 37°C samples is probably due to partial 15 stabilization of the gel structure by a few covalent bonds. It has been reported by Michon et al. (Rheol. Acta 32: 94 - 103, 1993) that the gel point of 4% gelatin at 27°C is greater than or equal to 250 minutes. Earlier experiments (Bishop et al., Biochemistry 29:1861- 20 1869, 1990) showed that activation of factor XIII by thrombin (10 U thrombin/1 mg factor XIII) is complete in 15 minutes. The long lag period for the increase in viscosity is believed to reflect the formation of adhesion zones during gelation, which are essential for 25 the occurrence of factor XIII - catalyzed cross-linking.

#### Example 2

A 6.6% w/v gelatin mixture in 100 mM NaCl was allowed to swell for three hours, then dissolved at 60°C. 30 20 ml of the 6.6% gelatin solution was combined with 1 ml H<sub>2</sub>O containing 146 mg EDC (1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide) adjusted to pH 5.0 with NaOH. Samples were incubated for two hours at 22°C to allow cross-linking to occur.

-35 Tensile strength (load at break) was determined using an Instron (Canton, MA) Model 4500 materials

tester. Aliquots of the gel were prepared in 2 cm diameter X 0.75 mm jigs for tensile strength testing. The jigs were designed so that axial displacement of the upper and lower members occurred without lateral displacement, thereby ensuring that upper and lower surfaces remained parallel during testing. Aluminum foil was bonded to the bonding surfaces of the jig to provide a substrate for the test sample. Plastic shims of precise thickness were placed between the upper and lower jig members to control guage length. Thicker guage lengths were found to reduce susceptibility to imperfections in the surface of the aluminum foil substrate or misalignments of the jig members. The lower jig member was secured in a base clamp, and the upper member was secured by a clamp mounted to the transducer by means of a universal joint. The transducer was moved upwards, measuring force and distance until a break occurred. All measurements were recorded automatically. Results are shown in Table 1.

20

Table 1

Sample <u>(n=6)</u>	Load at Break <u>(kg/3.14 cm<sup>2</sup>)</u>
Control	2.050 ± 0.540
+EDC	3.214 ± 0.300

Example 3

30 The effects of cross-linking protamine sulfate and fibronectin into gelatin were investigated. All samples contained a final concentration of 4% gelatin in 25 mM Tris pH 7.4, 100 mM NaCl, 3 mM CaCl<sub>2</sub>, 0.2% NaN<sub>3</sub>. Control samples contained gelatin only, gelatin plus 0.4 mg/ml human fibronectin (obtained from Alpha Therapeutics, Los Angeles, CA and chromatographically purified on DEAE resin to remove albumin), or gelatin

plus 0.4 mg/ml protamine sulfate (Salmon grade X, Sigma Chemical Co., St. Louis, MO). Experimental samples further contained 2 mg/ml recombinant human factor XIII. Factor XIII was activated at the start of the experiment by the addition of 1 unit/ml of bovine thrombin. The samples were incubated at 37°C for one hour and allowed to gel for 2 hours. Compressive testing of the samples was performed with an Instron Model 4500 materials tester equipped with a 5 mm probe. The probe was pushed into a 1.5 mm diameter contained gelatin sample until a break was recorded. Results are shown in Table 2.

Table 2

	Sample <u>(n=5)</u>	Max. Load (kg/0.196 cm <sup>2</sup> )	Energy to Break (kg/mm/0.196 cm <sup>2</sup> )
15	Control	0.054 ± 0.021	0.176 ± 0.019
	+FXIII	0.077 ± 0.016	0.319 ± 0.042
	+FN	0.065 ± 0.098	0.125 ± 0.030
	+FN, +FXIII	0.087 ± 0.026	0.620 ± 0.189
20	+Prot.	0.063 ± 0.019	0.174 ± 0.025
	+Prot., +FXIII	0.063 ± 0.010	0.166 ± 0.028

FXIII, factor XIII; FN, fibronectin; Prot., protamine sulfate

25 The maximum load at breaking was not substantially different for between control and factor XIII samples, but the energy to break for the +FN, +FXIII sample was greater than 2.4 times that of the control.

30 Example 4  
Gelatin (10% by weight) in 350 ml of 25 mM Tris pH 7.4, 100 mM NaCl, 3 mM CaCl<sub>2</sub> was allowed to swell for two hours at room temperature and then dissolved by incubating at 37°C in a water bath. The solution was returned to room temperature for at least two hours to

allow it to gel. Viscosity of the gel, as determined using a cone and plate viscometer (Brookfield), was 6 cp. The gel was remelted at 37°C and allowed to gel again, after which the viscosity was determined to be 12 cp.

5

Example 5

330 ml of 10% gelatin in 25 mM Tris pH 7.4, 100 mM NaCl, 3 mM CaCl<sub>2</sub>, 0.2% NaN<sub>3</sub> was allowed to swell for two hours at room temperature and then dissolved at 37°C in a water bath. The solution was gelled at room temperature in a water bath and remelted at 37°C. The solution was diluted to 6.6% gelatin by adding 149.1 ml of 25 mM Tris pH 7.4, 100 mM NaCl, 3 mM CaCl<sub>2</sub>, 0.2% NaN<sub>3</sub>. To 239.6 ml of the diluted solution was added 18.9 ml of recombinant human factor XIII (13.2 mg/ml in glycine/EDTA/sucrose storage buffer) or 18.9 ml of buffer alone, plus 1.0 ml of bovine thrombin (500 U/ml in 25 mM Tris, pH 7.4). Each sample was divided into six 40 g aliquots, which were placed in 50 ml beakers sealed with Parafilm and incubated at 21°C for 20 hours.

20  
25  
The gels were placed in an Instron (Canton, MA) Model 4500 materials tester. A 12 mm probe coupled to a 100N cell was run into the gel at 17 mm/minute. Measurements were continued until the gel failed. All control gels had an initial partial failure followed by a complete failure. The initial failure was not observed in the factor XIII cross-linked gels. Test results are shown in Table 3.

30

Table 3

<u>Sample</u>	Load at Break (kg/1.13 cm <sup>2</sup> )	Young's Modulus (kg/cm <sup>2</sup> /mm)
6.6% gelatin	0.557 ± 0.112	0.046 ± 0.004
6.6% gelatin		
+ factor XIII	1.469 ± 0.183	0.159 ± 0.030

Example 6

5 A 4% gelatin solution in 25 mM Tris pH 7.4, 100 mM NaCl, 3 mM CaCl<sub>2</sub>, 0.2% NaN<sub>3</sub> was prepared as described  
in Example 5. Samples were prepared in 2 cm diameter X  
0.75 mm tensile jigs. Factor XIII was added to test  
samples, and all samples were incubated for 20 hours at  
room temperature. Tensile strength was determined  
essentially as described in Example 2. Results are shown  
10 in Table 4.

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Table 4

Sample (n=5)	Load at Break (kg/3.14 cm <sup>2</sup> )
control	1.192 ± 0.305
+factor XIII	2.196 ± 0.550

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20 All breaks appeared to be adhesive (the gel did  
not break internally but pulled away from the aluminum  
foil that was bonded to the jig) in both controls and  
factor XIII-containing samples. Because a linear  
relationship exists between gelatin concentration and  
strength (Fig. 2), results in Table 4 can be extrapolated  
25 to 1.97 and 3.62 kg/3.14 cm<sup>2</sup> for non-cross-linked and  
cross-linked 6.6% gels, respectively. These values are  
comparable to those for EDC cross-linked gels (Example  
2).

30 Example 7

The melting temperatures of gels prepared using  
various concentrations of factor XIII were determined.  
Type A gelatin (Ferrosan International A/S) was dissolved  
in 25 mM Tris pH 7.4, 100 mM NaCl, 3 mM CaCl<sub>2</sub> to a  
concentration of 6.6%. The indicated amounts of factor  
XIII and bovine thrombin (1 U/ml) were added to the

gelatin solution, and the mixture was incubated at 37°C for 15 minutes. The gelatin was then allowed to gel and cross-link at room temperature for 18 hours. Melting temperatures were determined by observation of 1 ml of gelatin after 30 minutes at the specified temperature. Results are shown in Table 5.

Table 5

	<u>Factor XIII (<math>\mu</math>g/ml)</u>	<u>Melting Point</u>
10	0	27-37°C
	62	27-37°C
	125	37-45°C
	188	37-45°C
15	250	45-65°C
	375	65-95°C
	500	>100°C
	1000	>100°C

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From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. For example, the physical and chemical characteristics of gels can be varied as required for particular pharmaceutical or industrial applications. Accordingly, the invention is not limited except as by the appended claims.

25  
30

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Bishop, Paul D.  
Lasser, Gerald

(ii) TITLE OF INVENTION: CROSS-LINKED PROTEIN GELS AND METHODS OF  
MAKING THEM

(iii) NUMBER OF SEQUENCES: 1

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(F) ZIP: 98102

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Parker, Gary E.  
(B) REGISTRATION NUMBER: 31,648  
(C) REFERENCE/DOCKET NUMBER: 95-35PC

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## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Leu	Ser	Gln	Ser	Lys
1			5	

Claims

We claim:

1. A method of preparing a cross-linked protein gel comprising:

    adding a transglutaminase to a composition of a temperature-sensitive gel-forming protein; and

    incubating said composition and transglutaminase at a temperature at which a like composition lacking transglutaminase will gel, whereby said composition is converted to a cross-linked gel.

2. A method according to claim 1 wherein said composition is in the form of an aqueous solution.

3. A method according to claim 1 wherein said composition is in the form of a gel.

4. A method according to claim 1 wherein said temperature-sensitive gel-forming protein is gelatin or collagen.

5. The method of claim 1 wherein the transglutaminase is selected from the group consisting of factor XIII, tissue transglutaminase, keratinocyte transglutaminase, epidermal transglutaminase, and prostate transglutaminase.

6. The method of claim 1 wherein the transglutaminase is a human transglutaminase.

7. The method of claim 1 wherein said transglutaminase is factor XIII.

8. The method of claim 1 wherein said transglutaminase is a microbial transglutaminase.

9. The method of claim 1 wherein said transglutaminase is a bacterial or fungal transglutaminase.

10. The method of claim 1 wherein said transglutaminase is an Oomycete transglutaminase.

11. The method of claim 1 wherein said composition further comprises one or more additional proteins selected from the group consisting of fibronectin, von Willebrand factor, vinculin, and laminin.

12. The method of claim 1 wherein said composition further comprises a cytokine or a hormone.

13. A method of preparing a cross-linked protein gel comprising:

forming an aqueous solution of a temperature-sensitive gel-forming protein;

adding transglutaminase to said solution in an amount sufficient to catalyze cross-linking of said temperature-sensitive gel-forming protein; and

incubating said transglutaminase-containing solution under conditions of time and temperature in which a like solution lacking transglutaminase will gel.

14. The method of claim 13 wherein said aqueous solution is subjected to at least one cycle of gelation and melting before the addition of said transglutaminase.

15. The method of claim 13 wherein the amount of transglutaminase is from 0.125 mg/ml to 2.0 mg/ml of said solution.

16. The method of claim 13 wherein said temperature-sensitive gel-forming protein is gelatin or collagen.

17. The method of claim 13 wherein said protein is gelatin and said transglutaminase-containing solution is incubated at a temperature of from 4°C to 33°C.

18. The method of claim 17 wherein the gelatin concentration is from 1% to 10% by weight and said transglutaminase-containing solution is incubated at a temperature of from 20°C to 32°C.

19. The method of claim 13 wherein said protein is gelatin, said transglutaminase is factor XIII, and said gelatin and factor XIII are present in a mass ratio of from 528:1 to 66:1.

20. The method of claim 13 wherein said transglutaminase-containing solution further comprises one or more additional proteins selected from the group consisting of fibronectin, von Willebrand factor, vinculin, and laminin.

21. The method of claim 20 wherein said one or more additional proteins is present in said transglutaminase-containing solution in an amount not exceeding 10% by weight of total protein in said gel.

22. The method of claim 13 wherein said transglutaminase-containing solution further comprises a cytokine or a hormone.

23. The method of claim 13 wherein said transglutaminase is a microbial transglutaminase.

24. A method of preparing a cross-linked protein gel comprising:

adding a transglutaminase to a temperature-sensitive protein gel, whereby said transglutaminase diffuses into the gel and catalyzes cross-linking of the protein.

25. A cross-linked protein gel prepared by the method of claim 1.

26. The cross-linked gel of claim 25, further comprising one or more additional proteins selected from the group consisting of fibronectin, von Willebrand factor, and laminin, and vinculin.

27. The cross-linked gel of claim 26, wherein said one or more additional proteins is present in said transglutaminase-containing solution in an amount not exceeding 10% by weight of total protein in said gel.

28. The cross-linked gel of claim 25, further comprising a cytokine or a hormone.

29. The cross-linked gel of claim 25, wherein said protein is gelatin or collagen.

30. The cross-linked gel of claim 25 which is coated on a polymeric or paper support.

31. The cross-linked gel of claim 30 further comprising silver halide grains.

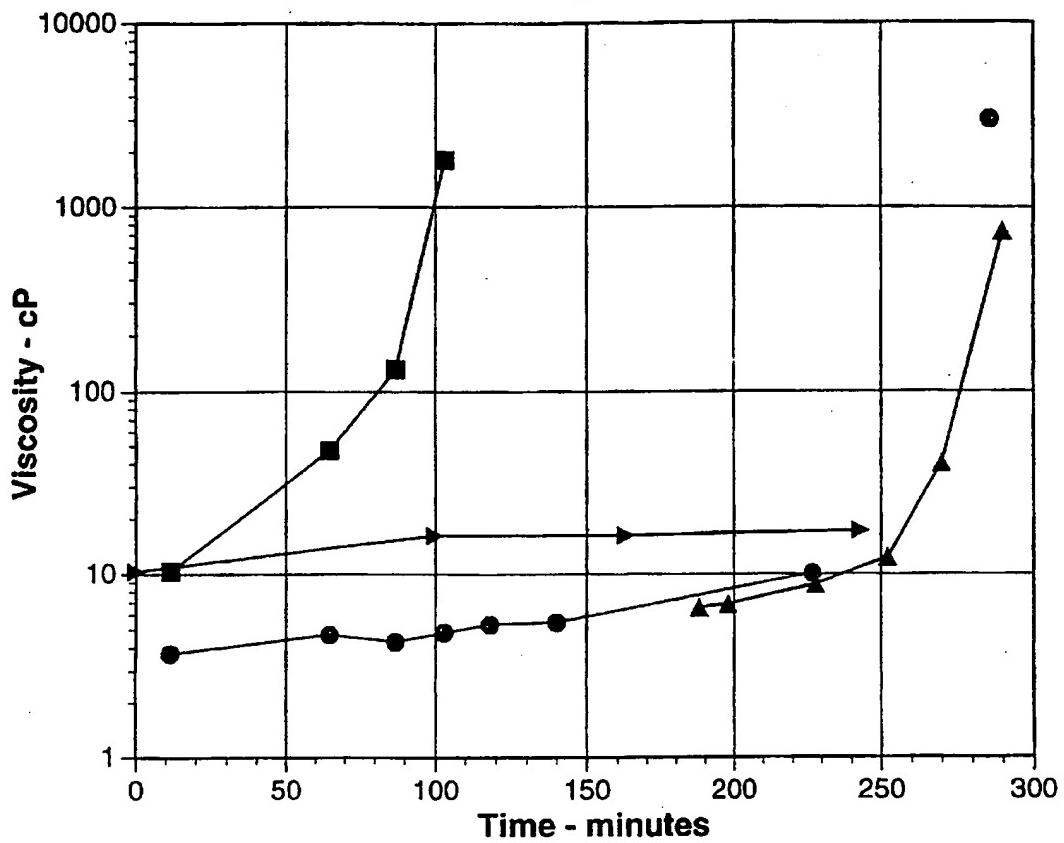
32. The cross-linked gel of claim 30 further comprising a dye-forming coupler compound.

33. The cross-linked gel of claim 25 which is in the form of a microcapsule.

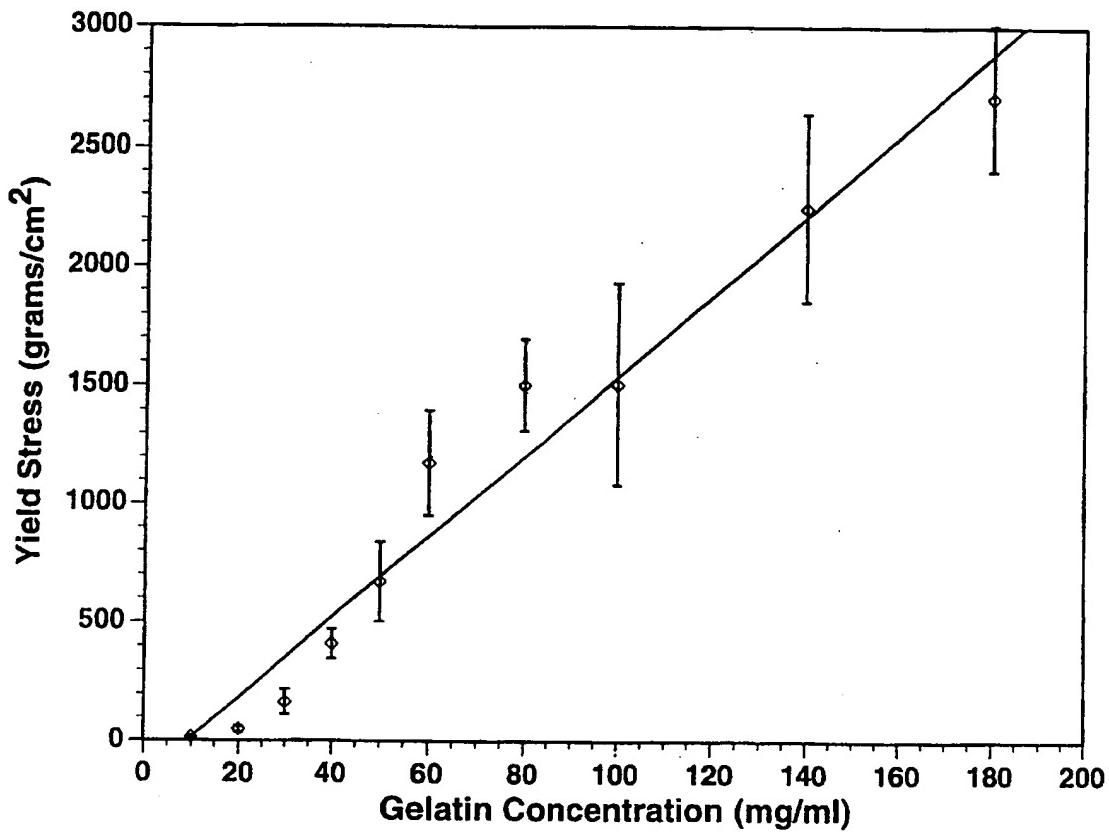
34. A pharmaceutical composition comprising a vehicle and an amine-containing drug, wherein said vehicle comprises a cross-linked gelatin or collagen gel prepared by the method of claim 1, and wherein said drug is covalently bonded to said gel.

1/2

Fig. 1



2/2

**Fig. 2**

# INTERNATIONAL SEARCH REPORT

Int'l Application No  
PCT/US 97/06605

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6 A23J3/06

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 A23J

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FOOD TECHNOLOGIST, vol. 23, no. 4, 1993, page 36-38 XP002038843 S.A. WILSON: "Cross linking of meat proteins for restructured products" see the whole document ---  X DATABASE WPI Section Ch, Week 8420 Derwent Publications Ltd., London, GB; Class D13, AN 84-123273 XP002038847 & JP 59 059 151 A (AJINOMOTO KK), 4 April 1984 see abstract --- -/-/	1
X		1,2

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

\* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
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Date of the actual completion of the international search	Date of mailing of the international search report
26 August 1997	15. 09. 97
Name and mailing address of the ISA  European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer  De Jong, E

## INTERNATIONAL SEARCH REPORT

Inte  
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**PCT/US 97/06605**

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